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14. ABSTRACT
Bone metastases are a debilitating and devastating complication for patients with advanced prostate cancer. Unfortunately, treatment options for patients with bone metastases are limited. Both hyaluronan synthase (HAS) and hyaluronic acid (HA) are upregulated in metastatic prostate cancer cells. 7-Hydroxy-4-Methyl Coumarin (HMC) is an inhibitor of hyaluronan synthase. It is commonly available in herbal supplements and, up to now, has been utilized mainly for digestion complaints. We propose that it may be efficacious in the prevention and treatment of prostate cancer. Our hypothesis is that hyaluronic acid (HA) is utilized by prostate cancer cells to facilitate metastasis. Thus, reducing the production of HA should reduce the metastatic potential of prostate cancer cells making HA an ideal target for preventing and treating metastatic disease. The goal of this current research proposal is to determine whether reduction of HAS, via treatment with HMC, will prevent prostate cancer metastasis to bone and other organs or serve as a viable treatment for established prostate cancer bone metastasis. To date, we have in vivo evidence that HA protein levels in vitro correlate with metastatic potential and that HA levels can be modulated in vitro using HMC. Furthermore, we have shown the in vitro growth of prostate cancer cells is slowed by inhibition of HA with HMC. We are currently completing the last of the in vitro studies and are beginning to assess the efficacy of HMC on in vivo tumor growth.

15. SUBJECT TERMS
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INTRODUCTION

Bone metastases are a debilitating and devastating complication for patients with advanced prostate cancer. Unfortunately, treatment options for patients with bone metastases are limited. Both hyaluronan synthase (HAS) and hyaluronic acid (HA) are upregulated in metastatic prostate cancer cells. 7-Hydroxy-4-Methyl Coumarin (HMC) is an inhibitor of hyaluronan synthase. It is commonly available in herbal supplements and, up to now, has been utilized mainly for digestion complaints. We propose that it may be efficacious in the prevention and treatment of prostate cancer. Our hypothesis is that hyaluronic acid (HA) is utilized by prostate cancer cells to facilitate metastasis. Thus, reducing the production of HA should reduce the metastatic potential of prostate cancer cells making HA an ideal target for preventing and treating metastatic disease. The goal of this current research proposal is to determine whether reduction of HAS, via treatment with HMC, will prevent prostate cancer metastasis to bone and other organs or serve as a viable treatment for established prostate cancer bone metastasis. To date, we have *in vivo* evidence that HA protein levels *in vitro* correlate with metastatic potential and that HA levels can be modulated *in vitro* using HMC. Furthermore, we have shown the *in vitro* growth of prostate cancer cells is slowed by inhibition of HA with HMC. We are currently completing the last of the *in vitro* studies and are beginning to assess the efficacy of HMC on *in vivo* tumor growth.

BODY

TASK 1: Determine whether hyaluronan synthase (HAS) expression and hyaluronic acid (HA) production in prostate cancer cells correlates with increased growth both *in vitro* and *in vivo* and whether modulation of HAS expression by 7-Hydroxy-4-Methyl Coumarin (HMC) will inhibit tumor growth in the primary (subcutaneous) site. (Months 1-12)

RESEARCH ACCOMPLISHMENTS:

a. Levels of HAS2 and HAS3 expression in established prostate cancer cell lines, PC-3, LN.CAP-LN3, VCaP, DuCaP, DU-145 and 22RV1 has been determined by quantitative expression analysis and compared to expression levels in non-tumor prostate epithelial cell lines, PZ-HPV-7 and RWPE-1. HAS2 and HAS3 levels were determined in each of the cells lines listed above by quantitative expression analysis. Results are shown in Figures 1 and 2 below. HAS1 expression was undetectable in the cancer cell lines. RNA from LN.CaP-LN3 cells was not available when these experiments were performed. We are in the process of repeating these experiments with this cell line included.

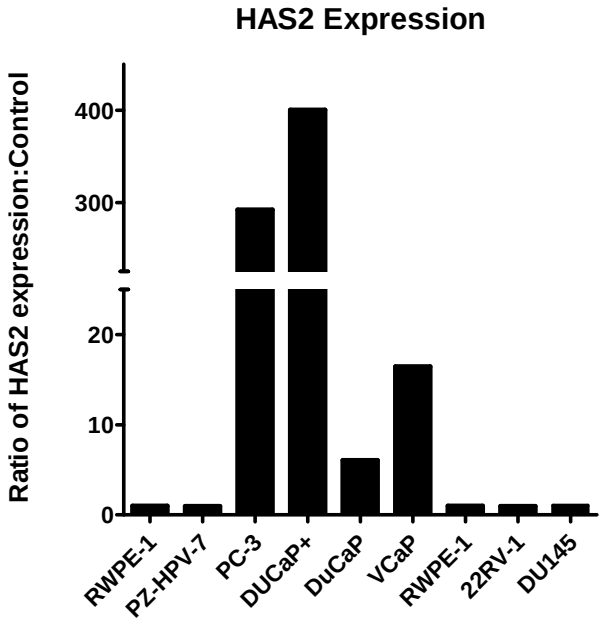

Figure 1. Quantitative Analysis of HAS2 Expression levels by Real-Time PCR. HAS2 expression is reported relative to expression in the RWPE-1 prostate epithelial cell line. Note that all of the prostate cancer cell lines express HAS2 at higher levels than prostate epithelial cell lines, RWPE-1 and PZ-HPV-7. DuCaP+ indicates DuCaP cells harvested with a feeder layer. DuCaP indicates only the isolated DuCaP cells.

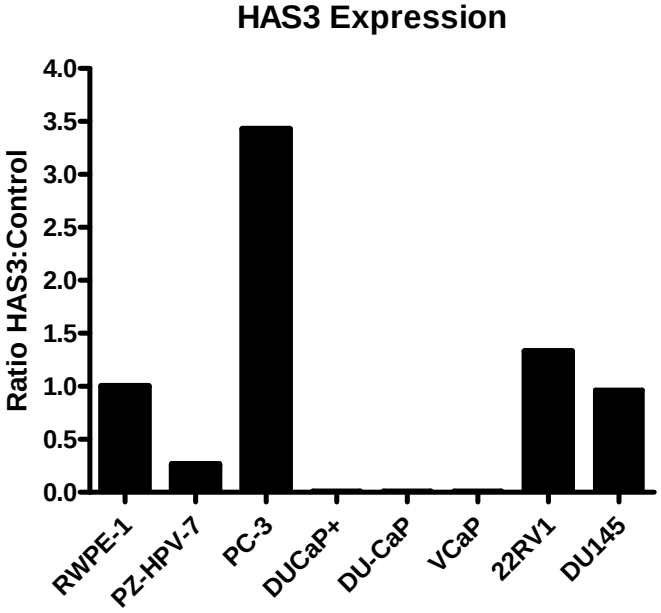

Figure 2. Quantitative Analysis if HAS3 Expression levels by Real-Time PCR. HAS3 expression is reported relative to expression in the RWPE-1 prostate epithelial cell line. Note that not all of the prostate cancer cell lines express HAS3. DuCaP+ indicates DuCaP cells harvested with a feeder layer. DuCaP indicates only the isolated DuCaP cells.

PC-3, the most aggressive of the prostate cancer cell lines, *in vivo*, expresses both HAS2 and HAS3 at much higher levels than the prostate epithelial cell lines, RWPE-1 and PZ-HPV-7 and the other prostate cancer cell lines. These differences are not nearly as remarkable as those observed with HAS2 expression. This indicates that HAS2 likely plays a much more important role in HA production in these cell lines. Interestingly, these results do not correlate with our initial studies which indicated increased HAS3 expression in DU-145 and VCaP cell lines as well. This data is currently being reproduced for verification and inclusion of LN.CaP-LN3 data.

b. HA synthesis was quantitated in the same cell lines examined in sub-task 1a using a competitive binding assay specific for HA. Again, non-tumor prostate epithelial cell lines, PZ-HPV-7 and RWPE-1 were utilized as controls (Figure 3). These results correlate with *in vivo* tumorigenicity and metastatic potential which has been previously determined in our laboratory.

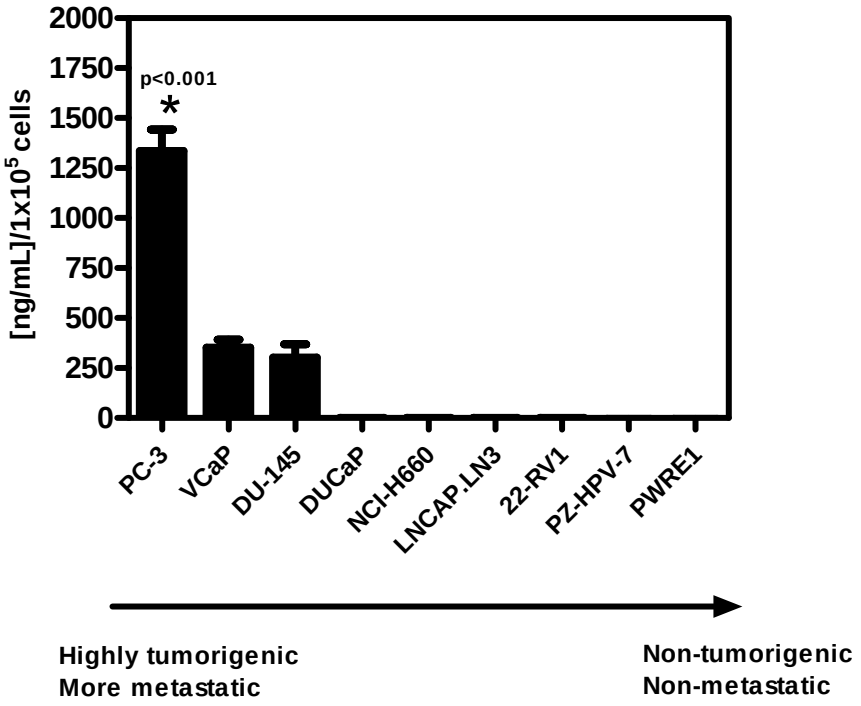

Figure 3. HA synthesis by prostate cancer cell lines. Note that the metastatic prostate cancer cell lines, PC-3, VCaP and DU-145 make HA. PC-3 cells make significantly more HA than other prostatic cancer cell lines and the prostate epithelial cell lines, PZ-HPV-7 and PWRE1. The other prostate cancer cell lines make very low levels or undetectable levels of HA. These results correlate with *in vivo* tumorigenicity and metastatic potential.

c. The prostate cancer cell lines were treated *in vitro* with HMC, a known inhibitor of HAS. Conditioned media was collected after 48 hours of incubation and quantitative expression analysis of HAS2 and HAS3 (Figures 4 and 5), HA synthesis (Figure 6) and *in vitro* growth rate (Figures 7-13) were examined in both HMC- and vehicle-treated cells. Cytotoxicity assays were performed using commercial colorimetric cell proliferation

assays (Promega), based on the cleavage of tetrazolium salts by mitochondrial dehydrogenases (MTS) in viable cells, but were uninformative due to interference of HA with the assay.

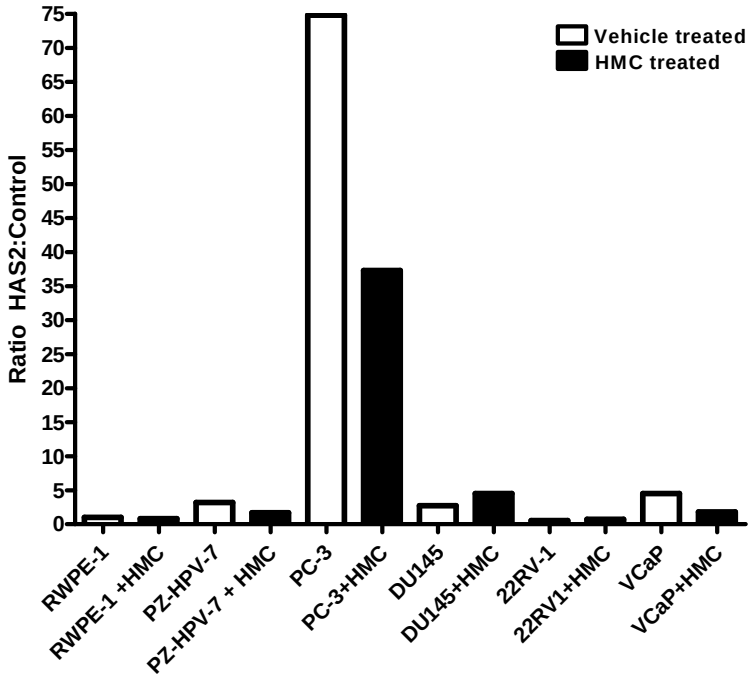

Figure 4. Quantitative Analysis of HAS2 Expression levels by Real-Time PCR. HAS2 expression is reported relative to expression in the vehicle treated RWPE-1 prostate epithelial cell line. HMC treatment reduced HAS2 expression levels in all of the cell lines except DU145. We are currently investigating the reasoning for this and are currently repeating this experiment to obtain data with DUCaP and LN.CaP-LN3 cells lines which were not available when this experiment was performed and to verify the other data.

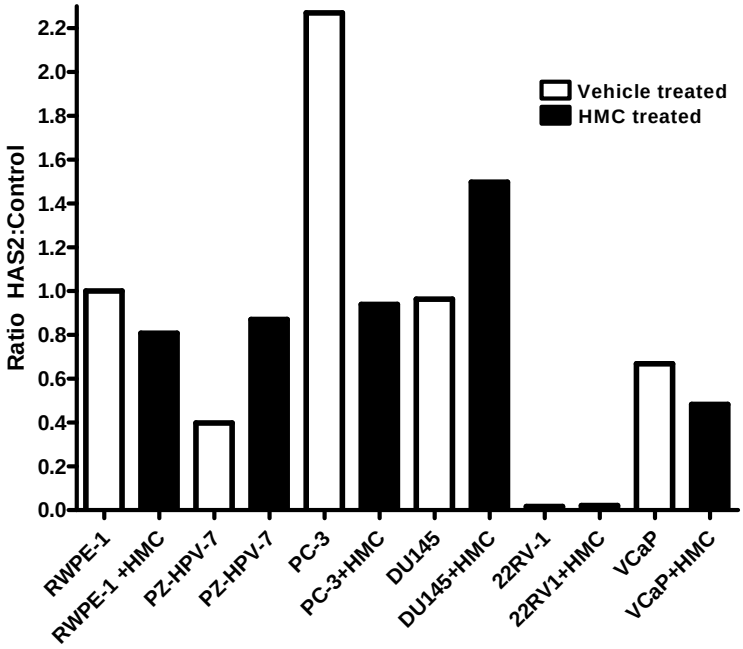

Figure 5. Quantitative Analysis of HAS3 Expression levels by Real-Time PCR. HAS3 expression is reported relative to expression in the vehicle-treated RWPE-1 prostate epithelial cell line. HMC treatment reduced HAS3 expression levels in all of the cell lines except DU145 and the prostate epithelial cell line PZ-HPV-7. We are currently investigating the reasoning for this and are currently repeating this experiment to obtain data with DUCaP and LN.CaP-LN3 cells lines which were not available when this experiment was performed.

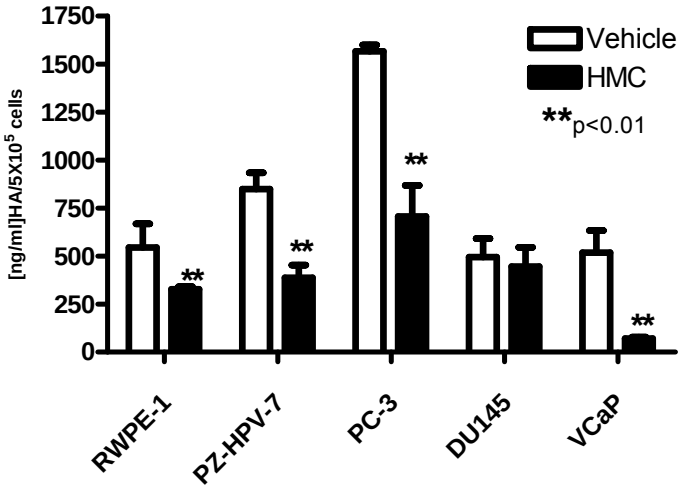

Figure 6. HA synthesis by prostate cancer cell lines. Note that the metastatic prostate cancer cell lines, PC-3, VCaP and DU145 make HA and thus were included in this experiment. HA production was significantly reduced in both PC-3 and VCaP but not in DU145 cells, consistent with the results of HAS2 and HAS3 expression analysis (Figures 4 and 5). The other prostate cancer cell lines make very low levels or undetectable levels of HA by this assay and thus were not included here.

These results are very interesting particularly since it appears that HA levels and HAS expression in DU145 may not be affected by inhibition of HAS by HMC. This warrants further investigation and will be explored outside the confines of this grant.

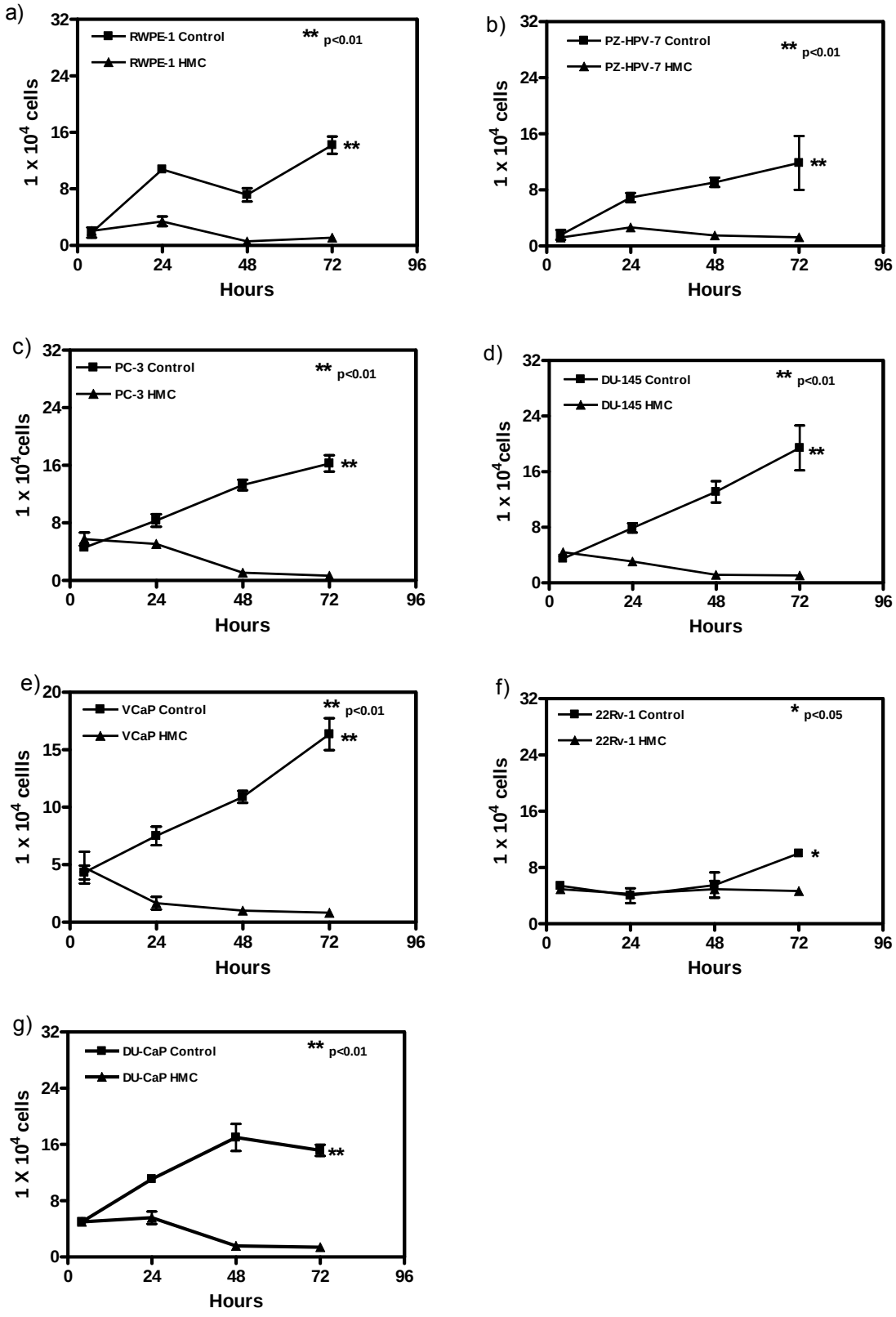

Figure 7 (a-g). ***In vitro*** **growth of vehicle-treated and HMC-treated prostate cancer cell lines.** Growth curves were generated by plating $1X10^4$ cells in media supplemented with either HMC or vehicle and counting at 4h, 24h, 48h, and 72h. The growth of all cell lines including nontransformed prostate epithelial cell lines, RWPE-1 and PZ-HPV-7, was significantly reduced.

d. To determine the safest dose of HMC in athymic nude mice, animals were divided into three groups and each group treated daily with a different concentration (100 mg/kg, 250 mg/kg and 500 mg/kg respectively) of HMC. This was to allow us to identify the highest dose that is well-tolerated by the animals.

   These experiments were performed using an acidic form of HMC. The mice tolerated this form reasonably well even at the highest dose proposed, even though the solution was thick and chalky. However, little if any change was seen in HA levels. After some discussion with Dr. Leach, the co-investigator on this grant, regarding the bioavailability of HMC, we scaled up on the doses of HMC and tested it at 3 higher concentrations (1.5g/kg, 2.5g/kg and 3.0g/kg). The bioavailability of HMC is very low and thus, an increase in dosage should increase the actual bioavailable HMC. Because the acidic form of HMC was difficult to get into solution, particularly so at higher concentrations, it was decided very recently to try to use the salt form of HMC. We have just finished testing this form in mice at the higher dose of 3g/kg/mouse/day, which has been used previously in other studies. This higher dose was well tolerated by the animals as were the lower doses. We plan to proceed with the remainder of the experiments using this form once we verify that there is a change in HA levels in our test mice. The data on HAS2 and HAS3 expression has already been confirmed using the salt solution of HMC. The remainder of the *in vitro* data is currently being repeated to verify that this form will be as effective as the acidic form on the growth of prostate tumor cells as well as to include the missing cell lines noted above. This work should be completed in the next 2 months.

e. Nude mice will be inoculated with each of the prostate cancer tumor cell lines (n=16 mice per cell line; 8 per treatment group) and treated with the optimal dose of HMC identified in part c above or with vehicle. (8 mice per group X 6 cell lines X 2 groups = 96 mice total X 2 experiments**)

   We are just beginning these experiments with the more soluble salt solution of HMC. We expect that we will see a significant difference in tumor growth and HA levels when mice are treated with HMC. We expect to complete this work in the next 4 months.

Outcome: Expression of HAS and HA production has been established and shown to correlate with *in vivo* tumorigenicity and metastatic capability. Dosage of HMC for *in vivo* studies has been established and effect of the compound on tumor growth will be determined. Two cell lines with the highest levels of HAS expression and HA production will be selected for further study on the effect of this compound on bone metastases. At present, we expect that PC-3 and either DU145 or VCaP prostate cancer cell lines will be selected for further analysis once we confirm our preliminary data.

Note: No work has begun on Task 2 to date. We expect to begin this work in approximately 3 months but first want to complete our initial analysis of the salt solution of HMC.

TASK 2: Determine whether inhibition of HAS expression and HA production with HMC will prevent metastases of prostate cancer cells to bone and other organs in mouse models of bone metastases. Mice will be pre-treated with HMC to mimic the clinical scenario whereby a patient is diagnosed with advanced prostate cancer and may be at risk for bone metastases, but has no evidence yet of skeletal lesions at diagnosis. HMC could then be used to augment standard of care to prevent metastases. **(Months 13-24)**

a. Athymic nude mice will be treated with the optimal dose of HMC or vehicle daily for two weeks prior to tumor cell inoculation (n=12 per group X 2 cell lines X 2 treatments X 2 experiments **for a total of plus 96 mice)
b. PC-3 and VCaP or DU145 prostate cancer cells will be treated with HMC or vehicle *in vitro* prior to inoculation into mice.
c. Mice will then be inoculated with either PC-3 cells or VCaP or DU145 cells via intra-cardiac inoculation. Treatment of the mice with the established dose of HMC or vehicle will continue on a daily basis for the duration of the experiment.
    a. Mice will be examined by radiography at baseline and then weekly beginning at 2 weeks post heart injection for the development of bone metastases in the case of PC-3, where skeletal lesions will develop more rapidly and most likely will be osteolytic in nature. VCaP and DU145 cells both cause a more osteoblastic phenotype and it will take longer for the development bone metastases so these mice will be x-rayed once per month until there is evidence of bone metastases in control animals and then more frequently. This will allow us to track the development of skeletal metastases over the course of the experiment.
    b. Serum samples will be harvested at baseline and once per month until sacrifice so that we can measure HA levels over the course of the experiment, as well as markers of bone turnover as indicators of bone metastases.
    c. Half of the mice in each group (n=6 per group X 2 cell lines X 2 treatments) will be examined by ^{18}F-FDG MicroPET at sacrifice for the identification of metastases to other organs
    d. At sacrifice, tissues from each mouse will be harvested for histological preparation. Quantitative bone histomorphometry will be performed on sections of long bones to determine the effects of HA on bone metastases. Sections will also be stained for HA as previously described, to examine the effect of HMC on tumor cell and host HA production in the bone metastatic site.

Outcome: We will have both radiographic and histological evidence in two *in vivo* models that targeting the production of HA using HAS is a viable treatment option to prevent prostate cancer metastases to bone and other organs.

Note: No work has been done on Task 3 yet. It is expected to be completed in Year 3 of this proposal.

TASK 3: Determine whether inhibition of HAS via treatment with HMC will be beneficial in animals with established prostate cancer bone metastases utilizing the same model used in Specific Aim 2. In this case, mice will be monitored radiographically for the development of bone metastases, and treatment with HMC will not begin until 75% of the mice have evidence of bone metastases. This would mimic the clinical scenario in which patients present with bone metastases at the time of diagnosis. (20 mice per group X 2 cell lines X 2 treatments X 2 experiments**160 mice total. [Note: more mice are used per group because of the nature of this experiment. Some animals may need to be euthanized before treatment begins and our analysis indicates that n=20 is the minimum number needed to observe a statistical difference.] **(Months 25-36)**

a. PC-3 and VCaP or DU145 prostate cancer cells will be treated with HMC or vehicle *in vitro* for 48 hours prior to inoculation into mice.
b. Athymic nude mice will then be inoculated with either PC-3 cells or VCaP or DU145 cells via intra-cardiac inoculation.
c. Mice will be examined by radiography at baseline and then weekly beginning at 2 weeks post heart injection for the development of bone metastases in the case of PC-3, where skeletal lesions will most likely be osteolytic in nature and develop more rapidly. VCaP and DU145 cells will have a more osteoblastic phenotype and take longer to develop bone metastases so these mice will be x-rayed once per month until there is evidence of bone metastases in control animals. This will allow us to track the development of skeletal metastases over the course of the experiment and to determine when approximately 75% of the mice have evidence of bone metastases.
d. Daily treatments with the optimal dose of HMC or vehicle will begin when 75% of the mice have evidence of bone metastases by radiography.
e. Serum samples will be collected at baseline and once per month until sacrifice so that we can measure HA levels over the course of the experiment as well as markers of bone turnover as indicators of bone metastases.

f. Half of the mice in each group (n=6 per group X 2 cell lines X 2 treatments) will be examined by ^{18}FDG MicroPET at sacrifice for the identification of metastases to other organs
g. At sacrifice, tissues from each mouse will be harvested for histological preparation. Quantitative bone histomorphometry will be performed on sections of long bones to determine the effects of HA on bone metastases. Sections will also be stained for HA to examine the effect of HMC on tumor cell and host HA production in the bone metastatic site.

Outcomes: We anticipate that these experiments will indicate that *in vivo* systemic treatment with HMC to treat established bone metastases may help reduce the progression of the bone metastases and thus HMC may be a viable treatment option for patients with already established bone metastases.

******Each *in vivo* experiment will be performed twice for reproducibility and consistency of results and 3 unmanipulated controls are always included in each experiment to ensure reliability of our techniques.

All of the experiments proposed here are designed to provide pre-clinical evaluation of HMC as a potential agent for the prevention and treatment of prostate cancer bone metastases. These are important experiments which could lead to clinical trials of HMC in patients with prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS:

- Levels of HAS1, HAS2 and HAS3 expression in prostate cancer cell lines has been determined. HAS1 expression is virtually undetectable in all of the cell lines. HAS2 expression is upregulated in PC-3, DuCaP, VCaP cell lines. HAS3 expression levels are substantially lower than HAS2 in all of the cell lines. HAS3 is expressed most abundantly in PC-3, 22RV1 and DU145 cells. This indicates that HAS2 is likely to be responsible for the bulk of HA production in prostate cancer cells.

- Levels of HA production by prostate cancer cells has been established and has been shown to correlate with tumorigenicity and metastatic behavior. Cell lines that have a more aggressive phenotype in mouse models, such as PC-3, VCaP and DU-145, produce more HA than other prostate cancer cell lines which are less aggressive. These three cell lines are also those initially isolated from the more aggressive cancers (metastatic to bone, bone and brain, respectively).

- Treatment with HMC *in vitro* has been demonstrated to decrease HAS 2 and HAS3 expression levels in prostate cancer cell lines. The exception to this is DU145, where expression increased slightly in both cases following treatment with HMC. We are currently repeating these experiments to verify this result.

- Treatment with HMC *in vitro* has been shown to decrease HA production as measured by ELISA in all of the prostate cancer cell lines that produced detectable levels of HA. Levels of HA in DU145 were reduced but the result was not significant. Again, we are currently repeating experiments to verify this result.

- Treatment with HMC *in vitro* resulted in a significant reduction in cell growth over time in all of the cell lines examined. This is consistent with anti-tumorigenic behavior. Interestingly, HMC significantly reduced the growth of DU145 cells despite our findings that it increased expression of HAS2 and HAS3.